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Original Article

Patulin and patulin producing *Penicillium* spp. occurrence in apples and apple-based products including baby food

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Abstract

Introduction: Patulin has raised the international attention because of its health risk. In fact, it has mutagenic, neurotoxic, immunotoxic, genotoxic and gastrointestinal effects in animals. In the present work, patulin and patulin-producing *Penicillium* spp. in apple and apple-based products marketed in Qatar were analysed.

Methodology: Sampling was carried out using apple fruits and apple-based products. Fungi were isolated from undamaged apples, apple juice and baby apple food. DNA extraction was carried out with DNeasy Plant Mini Kit (QIAGEN, Valencia, USA). The molecular identification of fungal isolates was carried out using *ITS1-ITS4 PCR.* PCR products were sequenced and blasted. Patulin was extracted and analyzed by LC/MS/MS, then quantified using Agilent 1290UHPLC coupled to 6460 triple quadruple mass spectrometer.

Results: Forty-five samples of undamaged fresh apple fruits, apple juice and apple-based baby food products sold in different markets in Qatar were surveyed for both fungal and patulin contamination using Liquid Chromatography Tandem Mass Spectrometery (LC/MS/MS). Twenty-five *Penicillium* spp. isolates were selected, including 23 *P. expansum* and one isolate each of *P. brevicompactum* and *P. commune*. All the tested *Penicillium* spp. isolates produced patulin *in vitro* (from 40 to 100 µg/g on Malt Yeast Extract agar medium). Patulin was detected in 100% of apple juice samples at levels ranging from 5.27 to 82.21 µg/kg. Only 5 samples contained patulin levels higher than European Union recommended limit (50 µg/kg). The average patulin contamination was 30.67 µg/kg and 10.92 µg/kg in baby apple juice and in baby apple compote, respectively.

Key words: Mycotoxin; *Penicillium*; food safety; fungal contamination.

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Introduction

Patulin is an α, β-unsaturated γ -lactone, mainly produced by species from the fungal genera *Penicillium, Aspergillus* and *Byssochlamys. Penicillium expansum*. It is known as the major producer of this mycotoxin [1] among other patulin-producing species belonging to the genus *Penicillium* [2]. This psychrophilic fungus is considered the most relevant agent of "blue mould'' spoilage in apples and causes significant economic losses worldwide. A number of studies have been focused on isolating fungal strains capable of producing patulin from different kinds of fruits and vegetables. In Canada, 24 strains of *P. expansum* were identified from an assortment of apples, among them 83% produced patulin at levels as high as 100×10^3 µg/L when they were cultured on Yeast extract-sucrose (YES) medium [3]. Also, the analysis of mycoflora contaminating apples marketed in Brazil, showed that the most prevalent fungal genus was *Penicillium* [4] with 94% and 60% of *P. expansum* and *P. griseofulvum* isolates, respectively, being able to produce patulin under the tested conditions.

Patulin has raised the international attention because of its health risk. In fact, this mycotoxin has mutagenic, neurotoxic, immunotoxic, genotoxic and gastrointestinal effects in animals [5,6]. Similar effects could be observed after long-term consumption of foods such as apple-based products contaminated by this toxin. For this reason, a maximum level of 50 µg/kg in fruit juices and fruit nectars marketed in Europe was set by the European Union (EU) (2003), while the maximum level allowed for apple products for infants and young children is only 10 µg/kg (European Commission, 2006). Because of patulin concern on

human health, a number of reports were published related to the occurrence of this compound in apple products. In China, the largest apple juice concentrate producer and exporter in the world, a total of 95 samples of apple products were analysed for patulin content and found to be contaminated within the range between 1.2 to 94.7 µg/L [7]. Moreover, 37%, of baby food in this report exceeded the EU permitted limit. Also, Barreira *et al.* [8] reported that 41% of the 68 apple juices marketed in Portugal were contaminated with patulin, with a maximum level of 42 µg/kg. In Spain, patulin was detected in 30 and 42 samples of apple juice and apple baby food, respectively, without exceeding the tolerable levels [9]. Limited research has been established to detect fungi producing patulin or to quantify the presence of patulin as a contamination factor in the Gulf area and particularly in Qatar. For such reason, we aimed to explore the presence of patulin and patulin-producing *Penicillium* spp. in apple and apple-based products marketed in Qatar. These results may represent a tool for the specialised authorities to evaluate the health risk posed by these imported products and to take measures aiming to reduce patulin risk for humans.

Methodology

Sampling

Forty-five samples of apple fruits and apple-based products including: 12 samples (1 kg each) of undamaged apple fruits, 20 samples of apple juice, 7 samples of baby apple compote and 6 samples of baby apple juice were collected at various retail outlets in Doha, Qatar, during 2013. The samples were immediately processed as described in detail below or stored at 4°C until processing.

Fungal isolation and storage

Undamaged apples were surface-disinfected with 1% NaClO solution for 1 min and rinsed three times with sterile distilled water. Using a sterile scalpel, apple tissue fragments (approximately 1 cm^3) were cut, plated on potato dextrose agar (PDA, Himedia, Mumbai, India) and incubated for 5 days at 28ºC. For apple juice and baby apple food, 1 mL from each sample was added to 9 mL sterile water and mixed thoroughly by shaking for 2 min. One-hundred µL were cultured on PDA, and then the plates were incubated for 5 days at 28ºC and examined for fungal contamination. After monospore isolation on potato dextrose agar (PDA; Himedia, Mumbai, India), fungal cultures were preserved in sterile water at 4°C until molecular identification [10].

Fungal DNA extraction

To perform genomic DNA extraction, fungi were grown for 3-7 days at 28°C in potato dextrose broth (PDB; Becton, Haryana, India). Fungal mycelia were collected from liquid medium using a sterile tip and ground in a mortar using liquid nitrogen. The DNA extraction was carried out with DNeasy Plant Mini Kit (QIAGEN, Valencia, USA) according to the manufacturer's instructions. Five µL of each DNA sample were checked on a 1% agarose gel containing ethidium bromide (0.5 µg/mL) and visualized under ultraviolet light.

Molecular identification of fungal isolates

ITS1-ITS4 PCR reaction

The 5.8S ribosomal DNA region (600 bp) of the selected isolates was amplified using universal primers ITS1 and ITS4 [11]. Each PCR reaction was performed using 12.5 µL of Taq PCR Master Mix 2X (QIAGEN, Valencia, USA), 0.5 µL of both the forward and reverse primers (10 pmol), 5 to 10 ng DNA template and ultrapure Water up to 25 µL. The thermal cycler (Life Technologies, New York, USA) was set as follows: initial denaturation at 94 °C for 5 minutes; 35 cycles of 30 seconds at 94 °C, 30 seconds at 54 °C and 30 seconds at 72 °C, followed by a final elongation step of 5 minutes at 72 °C. To check the amplified DNA, 5 µl of each reaction were loaded on a 1% agarose gel as described.

PCR product purification and sequencing

PCR products were purified with Pure-Link PCR Purification Kit (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions, and their concentration was then determined by Nanodrop (Thermo Scientific, Waltham, USA). Twenty ng of DNA template, 3.2 pmol of either ITS-1 or ITS-4 primer, 4 µL Ready Reaction Premix (Life Technologies, California, USA), 2 µL BigDye sequencing buffer (Life Technologies, Carlsbad, USA) and ultrapure water up to 20 µL were used in PCR reaction. The thermocycler was set as follows: initial denaturation at 96 °C for 1 minute; 25 cycles of 10 seconds at 96 °C, 50 seconds at 50 °C, and 60 seconds at 60 °C. Sequencing was carried out at Qatar University using the 3500 Genetic Analyzer (Life Technologies, New York, USA). Sequences were blasted in the National Center for Biotechnology Information (NCBI) database to provide species identification.

Isoepoxydon dehydrogenase gene amplification

The isoepoxydon dehydrogenase (*IDH*) gene region (600 bp) of the tested isolates was amplified using IDH1: 5′ CAATGTGTCGTACTGTGCCC 3′ and IDH2: 5′ACCTTCAGTCGCTGTTCCTC 3′ (Paterson, 2006). Each PCR reaction was performed using 12.5 µL of Taq PCR Master Mix 2X (QIAGEN, Valencia, USA), 0.5 µL of both the forward and reverse primers (10 pmol), 5 to 10 ng DNA template and ultrapure H_2O up to 25 µL. The thermal cycler (Life Technologies, New York, USA) was set as follows: initial denaturation at 96 °C for 2 minutes; 30 cycles of 30 seconds at 96 °C, 45 seconds at 52 °C and 90 seconds at 72 °C, followed by a final elongation step of 5 minutes at 72 °C. To check the amplified DNA, 5 µL of each reaction were loaded on a 1% agarose gel as described.

Patulin test production

For the determination of patulin production by *Penicillium* spp. isolates, the method described by Neri *et al.* [12] was used. Briefly, mycelial plugs of each isolate were cultured on Malt Yeast Extract agar medium (MEA) (Scharlab, Barcelona, Spain). After 7 days of incubation at 20 °C, five 6 mm agar plugs of mycelia cut in different sites of the colonies and the total weight was determined. The extraction was done using 2 mL of ethyl acetate/formic acid (200:1, v/v) for 60 min. After centrifugation, the extract was evaporated to dryness in a rotary vacuum concentrator. The dried residue was re-dissolved in 1 mL of methanol for 30 min and filtered through a 0.45 µm filter (Supelco, Sigma-Aldrich, Taufkirchen, Germany) before liquid chromatography tandem mass spectrometery (LC/MS/MS) analysis. For each strain, three biological repeats were performed. The standard deviation (SD) was calculated based on the average of the three values.

Patulin extraction

The liquid-liquid method was used for patulin extraction [13]. Five mL of fresh apple juice or commercial apple juice sample were mixed with 20 mL of ethyl acetate. The flask was introduced in the sonication bath and allowed to extract for 15 minutes. The ethyl acetate phase was separated and transferred to another flask and the extract was evaporated using a rotary vacuum concentrator. When the residue was completely dry, it was immediately dissolved in 1 mL of methanol/water/acetic acid (83.5:16:0.5, v/v/v) solution and analysed by LC/MS/MS.

Patulin quantification method

The patulin quantification was done in the Central laboratories Unit of Qatar University using Agilent 1290UHPLC coupled to 6460 triple quadruple mass spectrometer machine according to the method of Rudrabhatla *et al.* [14]. A sample volume of 10 µL was injected. A column Nova-Pak C18 4 µm, 150 mm x 3.9 mm (Waters, Milford, USA) was used. LC separation was carried out using 0.1% acetic acid in 10% methanol in water buffer as mobile phase A, and 0.1% acetic acid in 100% methanol as mobile phase B. The gradient program was started with 100% to 15% of Mobile phase A throw 5 minutes then rapidly to 100% of mobile phase B for 1 minute to flash the column, using constant flow rate at 0.25 ml/min. Total run time was 6 minutes. Patulin peak appears at 1.7 min as retention time. The ESI-MS conditions were as follows: the drying nitrogen gas temperature was set at 250°C and the gas was introduced into the capillary region at a flow rate of 10 mL/min; the capillary was held at a potential of 4500 V

Table 1. Fungal and patulin contamination of fresh undamaged apple fruit imported in Qatar.

Matrix	Cultivar variety	Number of *Penicillium* spp. isolates	Patulin content average (µg/Kg) ± SD
Apple 1	Red Prince	3	ND
Apple 2	Royal Gala	2	ND
Apple 3	Cripps Pink	6	ND
Apple 4	Royal Gala	0	ND
Apple 5	Unknown	1	ND
Apple 6	Granny Smith	5	5.02 ± 0.41
Apple 7	Mutsu	1	3.85 ± 0.5
Apple 8	Royal Gala	0	ND
Apple 9	Red Delicious	0	5.62 ± 1.56
Apple 10	Granny Smith	0	ND
Apple 11	Royal Gala	3	12.67 ± 0.61
Apple 12	Scifresh	4	17.35 ± 4.17

SD: Standard deviation. The standard deviation (SD) was calculated based on the average of the three values. ND: Not detected (below detection limit).

relative to the counter electrode in the negative-ion mode. The fragmentor voltage was 135 V. When the MS was conducted in the MRM mode using first quadruple (Q1) at precursor ion 153.1 *m/z* polarity mode negative, then third quadruple (Q3) at 109 and 81 *m/z* as products ions at collesion Energy Voltage 7 and 10 respectively, at Dwell Time 0.3 sec. The patulin standard was purchased from Sigma-Aldrich (Taufkirchen, Germany). The calibration curve obtained for concentrations ranging from 1 to 1000 µg/L showed good linearity with a coefficient of determination (r^2) of 0.999, limit of Quantification (LOQ) is 5 µg/kg and Limit of detection (LOD) is 1 µg/kg. For each sample, three biological repeats were performed. The standard deviation (SD) was calculated based on the average of the three values.

Results

Fungal and patulin contamination of fresh undamaged apple fruit imported in Qatar

In this study, a total of 45 samples, including healthy fresh apple, apple juice, baby apple juice and baby apple compote, were analyzed for both the presence of patulin fungal producers and for patulin contamination. As expected, all apple juice and apple baby food samples were free from fungal contamination, in accordance to previous reports [15,16]. On the contrary, we were able to obtain 25 *Penicillium* spp. isolates from the internal tissues of apparently healthy apple fruit (Table 1).

Molecular and biochemical characterization of Penicillium spp. isolates obtained from undamaged fresh apples

The molecular identification of our fungal collection using the ITS1-ITS4 primers [11] allowed classifying 23 isolates as *P. expansum* and one isolate each as *P. commune* and *P. brevicompactum* (Table 2). *P. expansum* is mainly associated with blue mould rot and it is known as the major postharvest disease of apple worldwide [17]. The molecular screening was carried out as well through the detection of a 600-bp fragment of the isoepoxydon dehydrogenase (*IDH*) gene [18] which plays a key role in the metabolic pathway of patulin production. All the tested isolates resulted in positive amplification of the *IDH* target sequence (Table 2), but their ability to produce patulin needed to be confirmed on a patulin-inducing medium.

Table 2. Molecular and biochemical characterization of *Penicillium* spp. isolates obtained from undamaged fresh apples.

Isolate code	Matrix	Fungal species	GenBank accession number	Patulin content average (µg/g of MEA) ± SD
Pexp11	apple 1	*P. expansum*	*KJ933308*	33.2 ± 1.0
Pexp12	apple 1	*P. expansum*	*KJ933307*	110 ± 7.8
Pexp13	apple 1	*P. expansum*	*KJ933306*	50.2 ± 3.6
Pexp21	apple 2	*P. expansum*	*KJ933305*	29.5 ± 0.2
Pexp22	apple 2	*P. expansum*	*KJ933304*	21.6 ± 2.0
Pexp31	apple 3	*P. expansum*	*KJ933303*	22.9 ± 1.0
Pexp32	apple 3	*P. expansum*	*KJ933309*	30.1 ± 7.0
Pexp33	apple 3	*P. expansum*	*KJ933302*	44.9 ± 6.5
Pexp34	apple 3	*P. expansum*	*KJ933301*	64.0 ± 0.9
Pexp36	apple 3	*P. expansum*	*KJ933300*	59.2 ± 1.0
Pbrev37	apple 3	*P. brevicompactum*	*KJ933299*	4.26 ± 0.1
Pcom52	apple 5	*P. commune*	*KJ933298*	12.4 ± 1.0
Pexp61	apple 6	*P. expansum*	*KJ933297*	43.2 ± 3.2
Pexp62	apple 6	*P. expansum*	*KJ933296*	31.3 ± 2.8
Pexp63	apple 6	*P. expansum*	*KJ933295*	29.5 ± 2.2
Pexp64	apple 6	*P. expansum*	*KJ933294*	24.7 ± 3.1
Pexp65	apple 6	*P. expansum*	*KJ933293*	24.3 ± 1.0
Pexp71	apple 7	*P. expansum*	*KJ933292*	46.1 ± 3.4
Pexp111	apple 11	*P. expansum*	*KJ933291*	41.1 ± 2.6
Pexp112	apple 11	*P. expansum*	*KJ933290*	67.3 ± 6.9
Pexp113	apple 11	*P. expansum*	*KJ933289*	51.4 ± 6.1
Pexp121	apple 12	*P. expansum*	*KJ933288*	25.5 ± 1.4
Pexp122	apple 12	*P. expansum*	*KJ933287*	32.1 ± 3.7
Pexp123	apple 12	*P. expansum*	*KJ933286*	41.7 ± 0.5
Pexp124	apple 12	*P. expansum*	*KJ933285*	53.8 ± 0.8

Isolates were grown on Malt Yeast Extract agar medium (MEA) for 7 days at 20°C before patulin extraction. SD: Standard deviation; MEA: Malt Yeast Extract agar medium. The standard deviation (SD) was calculated based on the average of the three values.

Table 3. Incidence of patulin in fresh apples and apple-based products.

Sample category	Number of tested samples	Number of positive samples	Patulin range (µg/Kg)	Average patulin content (µg/Kg) ± SD	Number of samples exceeding> EU limit
Fresh apple	12	5	ND - 17.3	3.71 ± 0.6	0
Apple juice	20	20	5.8 - 82.2	35.37 ± 1.66	5
Baby apple juice	6	6	7.7 – 61.3	30.67 ± 6.7	3
Baby apple compote	7	7	1.02 – 24.57	10.92 ± 1.21	3

SD: Standard deviation. The standard deviation (SD) was calculated based on the average of the three values. EU: European union (37). ND: Not detected (below detection limit).

Incidence of patulin in fresh apples and apple-based products.

In the current study, 20 samples of imported apple juice were analyzed for patulin contamination. As shown in Table 3, all the samples resulted positive (average patulin content being 35.4 µg/kg) and five presented a patulin concentration over the maximum recommended limit in the EU. In 2010, Jalali *et al.* [19] observed the same percentage of patulin contamination in apple juice in Iran, with a mean of 30 µg/L and 13 % at levels exceeding 50 µg/L. In Belgium, 29 local and 14 imported apple juices were assayed for patulin. Almost 80 and 43%, respectively, of the tested samples were positive for this mycotoxin, without exceeding the EU permitted level [20,21].

Discussion

In Qatar, a wide range of apple varieties as well as apple-derived products are imported from different supply origin to the local market [22,23]. Given the strong dependence of the Gulf Region on food imports, food security issues are largely debated in these states [24,25]. Reports related to apple fruit and apple-derived product contamination by patulin have increased in recent years worldwide [26-31], but little information exist on the presence of this mycotoxin in fruit and derivate products imported to Qatar.

As expected, all apple juice and apple baby food analyzed samples were free from fungal contamination, in accordance to previous reports [15,16]. On the contrary, we were able to obtain 25 *Penicillium* spp. isolates from the internal tissues of apparently healthy apple fruit (Table 1). In fact, the absence of visible blue rot symptoms does not necessarily indicate the absence of mould contamination in apples, since fungal growth is not always externally visible [32,33]. Internal fungal contamination can be the result of insect invasion or agricultural practices during the pre-harvest stage, resulting in the risk of patulin contamination in externally undamaged apple fruit.

The molecular identification of the fungal collection using the ITS1-ITS4 primers allowed classifying 23 isolates as *P. expansum* and one isolate each as *P. commune* and *P. brevicompactum*.

In addition, all the tested isolates resulted in positive amplification of the *IDH* gene (isoepoxydon dehydrogenase), but their ability to produce patulin needed to be confirmed on a patulin-inducing medium. Indeed, Paterson [34] reported a good correlation between presence and absence of the *IDH* gene and patulin production ability *vs* non-production, respectively. However, it was possible to identify patulin negative strains presenting the intact *IDH* gene [35]. Thereby, the present study allowed to validate the molecular results and indeed patulin was produced by 100% of the *Penicillium* spp. isolates at levels as high as 110 µg/g of fresh Malt Extract Agar (MEA) medium. Abramson *et al.* [3] observed similar percentage (83%) of *P. expansum* being able to produce patulin with an average of $31x10^3$ µg/mL using Yeast extract-sucrose (YES) agar medium. Welke *et al.* [4] noticed that 94 and 60% of 35 *P. expansum* and 5 *P. griseofulvum* tested isolates, respectively, proved patulin producers under inducing conditions. Fourty-one percent of the sampled fresh apples were contaminated by patulin, albeit without exceeding the maximum permitted limits (Table 1). Thus, our results confirm previous evidence that the absence of visible symptoms of apple fruit rot does not exclude the risk of patulin contamination. Indeed, Jackson *et al.* [36] reported the same finding and detected patulin from visibly undamaged apples at concentrations reaching 15.1 µg/L of cider when controlled storage conditions were maintained. In this context, the Food and Agriculture Organization of the United Nations (FAO) (2003), considered the storage of apples as a critical control point, having a great influence on the risk of patulin contamination.

With respect to the presence of patulin in apple-derived baby food, the results were more critical, as 50% of the tested samples exceeded the tolerable limit of 10 µg/kg (Table 3). Also, the average for the baby

apple juice was 30.7 µg/kg, which is more than 3-folds of the maximum permitted limit, hence representing a high risk for the consumer of these products. In China, patulin was detected in 19 samples of baby food including apple juice, apple sauce, and mixed fruit sauce with a maximum of 67.3 µg/L [7]. Moreover, Cano-Sancho *et al.* [9] reported that 42 samples among 124 of apple-based baby food contained patulin but no sample exceeded 10 µg/kg. The high incidence of patulin found in this study is indicative of the poor quality of fruit used in production. It is well known that the faulty quality apple fruit (*e.g.,* long-term stored, rotten or damaged) are always sent for juice processing industry. FAO (2003) considers that apple reception step is most determinant for patulin content in the final secondary product. According to the same organization, apple batches with more than 10% rotten fruit should be rejected due to the elevated concentrations of patulin putatively present in such batches. Even accepting batches for juice production with the lowest possible amount of damaged apple creates a high risk of contamination for healthy fruit. Moreover, it was reported that cleaning procedures and elimination of rotten tissue before pressing do not necessarily eliminate completely all the patulin present, as this toxin is highly soluble in water and diffuses to healthy tissue [37]. As we report, the presence of patulin-producing *Penicillium* spp. in healthy-looking apple fruit cannot be excluded and for this reason during product processing, practices must avoid all risk of fungal growth and patulin production.

Conclusions

This report is the first study carried out in Qatar aiming to survey the occurrence of patulin in imported apple fruit and apple-based products. Based on the results, we conclude that the incidence of patulin in apple juice does not represent a serious risk for the adult consumer since the mean is below the limit recommended by the EU. However, the significant contamination of apple-derived baby food (juice and compote) marketed in Qatar constitutes a matter of concern. We recommend that a high level of awareness of the protection of infant groups is needed and that strict measures to control the quality of baby apple food imported in the Gulf Countries must be taken.

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